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Neuraminidase Expressed by *Streptococcus pneumoniae* Desialylates the Lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*: a Paradigm for Interbacterial Competition among Pathogens of the Human Respiratory Tract

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Both *Neisseria meningitidis* and *Haemophilus influenzae* are capable of mimicking host structures by decorating their lipopolysaccharides with sialic acid. We show that a neuraminidase expressed by *Streptococcus pneumoniae* (NanA) is able to desialylate the cell surfaces of both these species, which reside in and possibly compete for the same host niche.

Several bacterial species mimic host structures by sialylation of cell surface components (6). Among the major pathogens originating in the human respiratory tract, both *Neisseria meningitidis* and some isolates of *Haemophilus influenzae* express a sialyltransferase that adds sialic acid α-2,3 linked to galactose as a terminal structure on their lipopolysaccharide (LPS) (5, 7). The addition of sialic acid promotes survival by decreasing the bactericidal effect of complement through interaction with factor H (15). For *N. meningitidis*, sialic acid is obtained from cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA), which only some strains are able to synthesize (9). For *H. influenzae*, sialic acid is obtained from environmental sources of 5-acetylneuraminic acid (Neu5Ac) (8).

Streptococcus pneumoniae (the pneumococcus), which is also a common member of the flora of the human upper respiratory tract, has been shown to cleave sialic acid-containing substrates with α-2,3 and α-2,6 linkages to galactose as well as those with α-2,6 linkages to *N*-acetylgalactosamine (16). The pneumococcus expresses several distinct neuraminidases, including NanA and NanB (1, 2). In some strains, there is also a *nanB* homolog, *nanC*, the expression and activity of which have not yet been described. It has been suggested that neuraminidase activity promotes colonization by exposing host cell receptors otherwise covered by sialic acid (19). In this study, we test the hypothesis that an additional target of pneumococcal neuraminidase is sialic acid attached to the cell surface of other members of the nasopharyngeal flora.

N. meningitidis strain N3 or nontypeable *H. influenzae* strain H122 was grown in the presence or absence of CMP-NANA or Neu5Ac, respectively (Table 1.) Western analysis revealed that growth in the presence of a source of sialic acid corresponded with the loss of the monoclonal antibody (MAb) 3F11 epitope, recognizing lacto-*N*-neotetraose, a terminal LPS structure to which sialic acid is added in both species (Fig. 1 and 2) (9, 10). The loss of this epitope was associated with the presence of a higher-molecular-weight band in proteinase K-treated lysates in silver-stained Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Treatment of *N. meningitidis* (up to 2×10^8 CFU) or *H. influenzae* (2×10^6 CFU) with purified neuraminidase obtained from *Clostridium perfringens* (50 mU/ml) (Sigma-Aldrich Co., St. Louis, Mo.) resulted in expression of the MAb 3F11 epitope and loss of the higher-molecular-weight band, confirming that the differences in the LPS were caused by sialylation.

The effect of the pneumococcus in vitro was tested by incubation of *N. meningitidis* or *H. influenzae* under conditions allowing for LPS sialylation with culture supernatants of *S. pneumoniae* grown to the mid-log phase in C+Y medium (17). Incubation of N3 or H122 for 30 min at 37°C with the supernatant fraction of growth medium from pneumococcal strain

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	*S. pneumoniae nan* gene[a]			Source or reference
		nanA	*nanB*	*nanC*	
N. meningitidis N3	MC58C3, type B unencapsulated mutant				12
H. influenzae H122	Nontypeable clinical isolate				This study
S. pneumoniae					
P2	R6, unencapsulated[b] mutant of D39	+	+	−	20
P394	Type 4 genome sequence strain	+[c]	+	+	14
P1247	D39Δ*nanA*	−	+	−	18
P1252	Type 21 clinical isolate	+	−	−	This study
P1253	P1252Δ*nanA*	−	−	−	This study

[a] The presence of *nanB* was determined by PCR and Southern hybridization. The presence of *nanC* was determined by PCR (data not shown). +, present; −, absent.

[b] The presence of capsule had no apparent effect on neuraminidase activity in this study.

[c] Secreted fragment due to frameshift mutation upstream of the C-terminal cell surface-anchoring domain.

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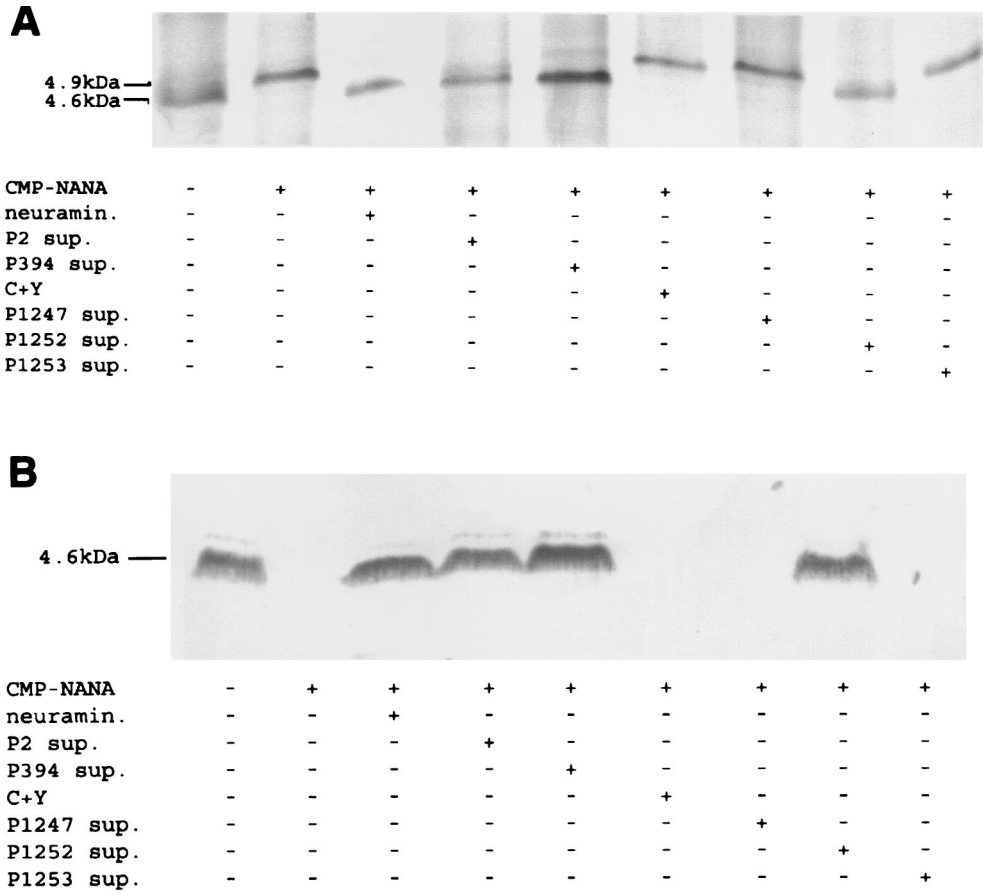

FIG. 1. Effect of pneumococcal neuraminidase on sialylation of meningococcal LPS. Meningococcal strain N3 was grown up in chemically defined media with or without CMP-NANA (50 μg/ml). A total of 2.0×10^8 CFU were then treated for 30 min at 37°C in C+Y medium with or without *C. perfringens* neuraminidase (neuramin.; 50 mU/ml) or supernatant (sup.) from 10^8 CFU of the *S. pneumoniae* strain indicated (13, 17). The bacterial pellet was incubated with proteinase K at 65°C and then separated in Tricine-SDS-PAGE gels. LPS was visualized with a modified silver stain (A) or transferred to a nitrocellulose membrane and immunoblotted with MAb 3F11 (B).

P2 or P394 (10^8 CFU/ml) resulted in loss of sialylation (Fig. 1 and 2). No effect was seen in control supernatants containing the C+Y growth medium alone. The addition of CMP-NANA (50 μg/ml) during incubation of N3 with the pneumococcal supernatants had no effect on desialylation, suggesting that under these conditions, the activity of the neuraminidase was more efficient than that of the meningococcal sialyltransferase (data not shown). The ability to desialylate the LPS of N3 and

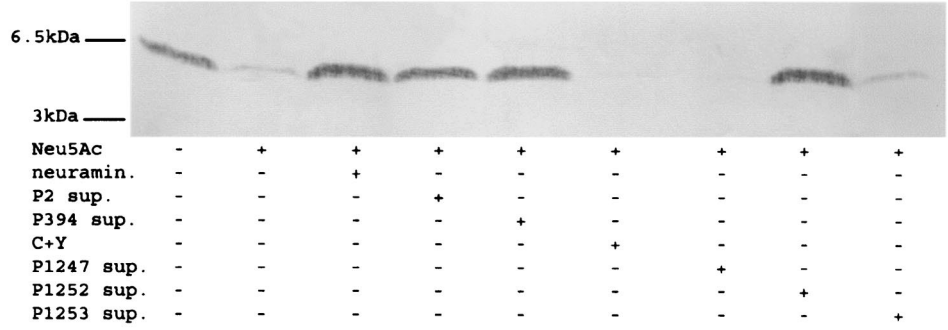

FIG. 2. Effect of pneumococcal neuraminidase on sialylation of *H. influenzae* LPS. *H. influenzae* strain H122 was grown on brain heart infusion agar supplemented with 1.5% Fildes enrichment (Difco Labs, Detroit, Mich.) with or without Neu5Ac (100 μg/ml). A total of 2.0×10^6 CFU were then treated for 30 min at 37°C in C+Y medium with or without *C. perfringens* neuraminidase (neuramin.; 50 mU/ml) or supernatant (sup.) from 1.0×10^8 CFU of the *S. pneumoniae* strain indicated. The bacterial pellet was lysed by treatment at 100°C for 5 min and then separated in Tricine-SDS-PAGE gels. LPS was visualized with a modified silver stain (data not shown) or transferred to a nitrocellulose membrane and immunoblotted with MAb 3F11.

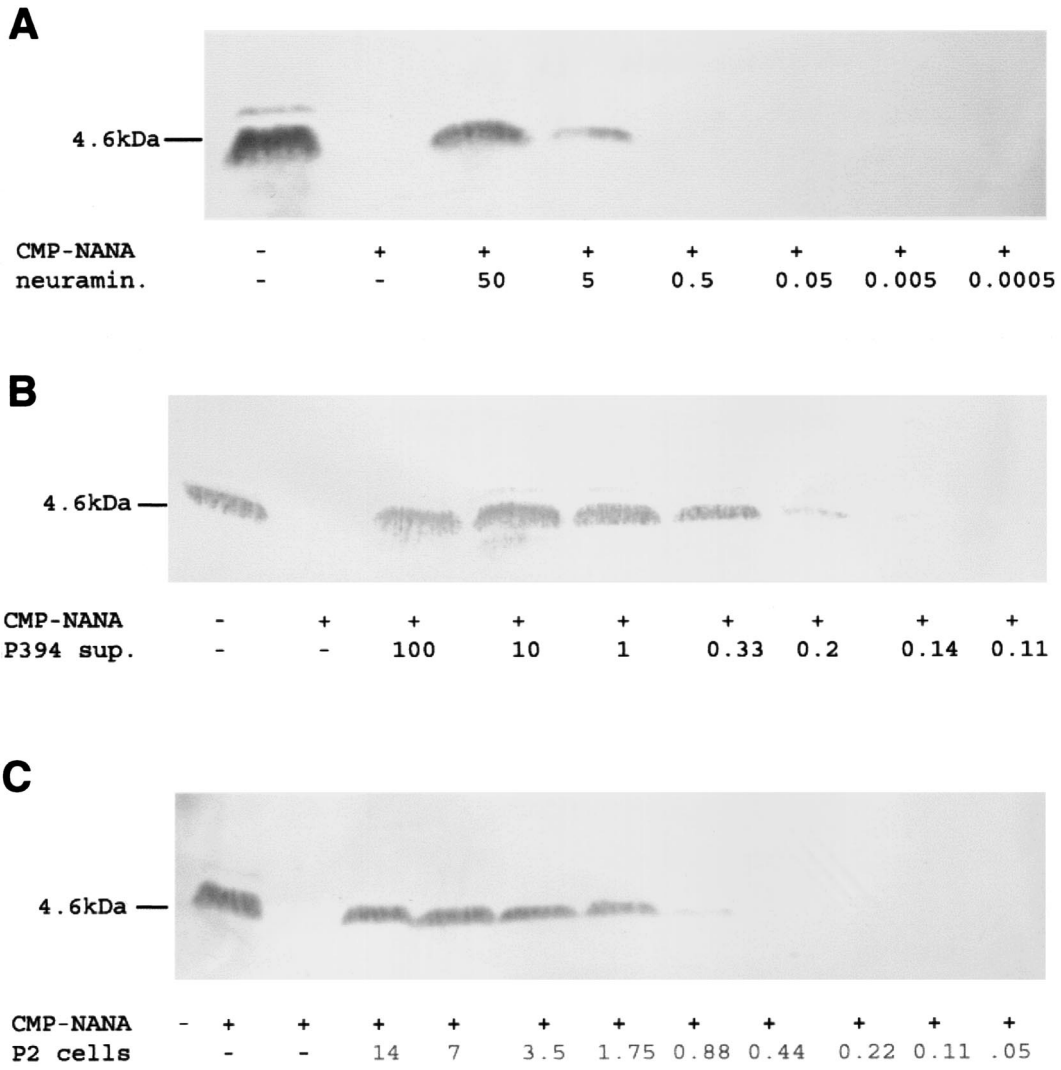

FIG. 3. Western analysis quantifying the neuraminidase activity of *S. pneumoniae* necessary for removal of sialic acid from meningococcal LPS. The absence of the MAb 3F11-reactive band indicates LPS sialylation. Meningococcal strain N3 grown with or without CMP-NANA (50 μg/ml) was treated as described above with purified *C. perfringens* neuraminidase (neuramin.) at the final concentration indicated (milliunits per milliliter) (A) or culture supernatant (sup.) from P394 cells at the cell density indicated (10^6 CFU) (B) or the cell fraction of P2 cells at the cell density indicated (10^7 CFU) (C).

H122 was noted in culture supernatant from strain P1252, but not that from P1247 or P1253, indicating that *nanA* is required for this activity (Fig. 1 and 2). The lack of activity in P1247 cells or culture supernatant, even when tested after growth to the stationary phase, when NanB expression is optimal, suggests that *nanB* does not contribute to the desialylation of the LPS (data not shown) (3). The neuraminidase activities of strains P2 (cell fraction) and P394 (culture supernatant fraction) were quantified by comparison to that of purified *C. perfringens* neuraminidase in serial dilutions (Fig. 3.) The results demonstrate that 5 mU of neuraminidase activity was sufficient for complete desialylation of 2×10^8 meningococci. The neuraminidase activity for strain P394 was estimated at 12 mU per supernatant fraction for 10^6 cells and 0.3 mU/10^6 cells for the cell fraction of strain P2. Thus, we estimate that the supernatant derived from one P394 cell is sufficient to desialylate about 1,000 meningococci under these conditions. In contrast, the activity of one P2 cell was sufficient to desialylate only about 25 meningococci. P394 contains a frameshift upstream of the C-terminal cell wall LPXTGX-anchoring motif in *nanA*, explaining why >95% of the enzyme activity was found in the culture supernatant rather than the cell fraction. Since the assay conditions would predict more efficient access of the enzyme to its target in the secreted form, the secretion of NanA in P394 could account for its higher level of activity. For P2, the neuraminidase activity was divided between the culture supernatant (28% of activity) and cell fractions (72% of activity), indicating partial release of the enzyme (data not shown).

We have previously shown that high concentrations of hydrogen peroxide produced by the aerobic metabolism of the pneumococcus may be inhibitory or bactericidal in vitro to other species that reside in the same environment, including *H. influenzae* and *N. meningitidis* (14). The findings in this study describe a second mechanism whereby *S. pneumoniae* could

interfere with the biology of potential competitors. In the case of desialylation of the cell surfaces of other members of the microflora, the pneumococcus appears to be specifically targeting a mechanism involving bacterial adaptation to its host. It remains to be determined whether interspecies competition occurs in the heavily colonized human upper respiratory tract in which each of the three species examined here resides. In this regard, several previous reports suggest that the pneumococcus may have inhibitory effects on *H. influenzae* in the natural host. During exacerbations of chronic bronchitis, *H. influenzae* was isolated less frequently during periods when the pneumococcus was present compared to periods when it was absent (11). Recent results from a randomized double-blind trial of the pneumococcal conjugate vaccine showed that a decrease in the incidence of carriage and otitis media caused by pneumococcal types in the vaccine was associated with an 11% increase in disease due to *H. influenzae* in the vaccine group (4). Disease caused by the meningococcus is less common, and we are not aware of a similar inverse association with the pneumococcus having been reported. Clinical observations about *S. pneumoniae* and *H. influenzae*, however, point out the need to understand the potential interactions of microorganisms, since manipulation of the human microflora may lead to unanticipated problems.

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